

**CLAIMS**

That which is claimed is:

- 5 1. A nucleic acid construct encoding a fusion protein, wherein the construct comprises a coding sequence for a protein of interest that is operatively linked to coding sequence for a prodomain protein, wherein the prodomain protein has an increased affinity for corresponding protease or variants thereof.
- 10 2. The nucleic acid construct according to claim 1, wherein the corresponding protease is subtilisin or a variant thereof.
- 15 3. The nucleic acid construct according to claim 2, wherein the prodomain protein further comprises amino acid sequences that increase binding affinity for subtilisin or variants thereof.
- 20 4. The nucleic acid construct according to claim 1, wherein the prodomain protein comprises a replacement sequence for the P1-P4 amino acid sequence comprising substitutions of amino acid residues F or Y for P4, any amino acid residue for P3, A or S for P2 and M, F, Y H, or L for P1.
- 25 5. The nucleic acid construct according to claim 2, wherein the prodomain protein is a prodomain of subtilisin.
- 30 6. The nucleic acid construct according to claim 5, wherein the prodomain protein comprises substitutions of amino acid residues F or Y for P4, any amino acid residue for P3, A or S for P2 and M, F, Y H, or L for P1 at the C-terminal end.
7. A fusion protein comprising a target protein operatively linked to a prodomain protein, wherein the prodomain protein is modified to exhibit an increased affinity for subtilisin or variants thereof
- 30 8. The fusion protein according to claim 7, wherein the prodomain protein is a subtilisin prodomain protein.

9. The fusion protein according to claim 8, wherein the subtilisin prodomain protein comprises an amino acid sequence of FKAM replacing P1 through P4 amino acids.

5 10. The fusion protein according to claim 7, wherein the prodomain protein comprises variations of amino acid residues E E D K L (F/Y) Q S (M/L/Y) to be used as a cognate sequence.

10 11. The fusion protein according to claim 7, wherein the target protein is staphylococcal Protein A<sub>B</sub> domain; Protein A<sub>B</sub> mutant A219; Streptococcal protein G<sub>B</sub> domain; Streptococcal protein G<sub>a</sub> domain; Protein G<sub>B</sub> mutant G311; E. coli hypothetical Yab; Bovine  $\alpha$ -subunit of transducin; M. thermautrophicus CDC6; streptavidin; avidin; Taq polymerase; alkaline phosphatase; RNase; DNase; restriction enzymes; peroxidases; endo-1,4-beta glucanase; endo-1,3-beta-glucanase; chitinases; beta and alfa glucosidases; beta and alpha glucuronidases; amylase; glucosyl-transferases; phospho-transferases; chloramphenicol-acetyl-transferase; beta-lactamase; luciferase; esterases; lipases; proteases; bacteriocines; antibiotics; enzyme inhibitors; growth factors; hormones; receptors; membranal proteins; nuclear proteins; transcriptional factors; translational factors or nucleic acid modifying enzymes.

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13. A method for the production of a subtilisin binding fusion protein, the method comprising:

25 providing a nucleic acid construct encoding a fusion protein wherein the fusion protein comprises a prodomain protein and a second protein of interest, wherein the prodomain protein is modified to bind subtilisin or variants thereof with high affinity;

transfecting a host cell with the nucleic acid construct; and

culturing the transformed host cell under conditions suitable for expression of the fusion protein.

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14. The method according to claim 13, wherein the prodomain protein is the prodomain of subtilisin.

15. The method according to claim 14, wherein the prodomain protein is modified by

replacing the P4 through P1 amino acids with amino acid sequences FKAM, FKAY or FKAF

16. The method according to claim 15, wherein the second protein of interest is  
5 staphylococcal Protein A<sub>B</sub> domain; Protein A<sub>B</sub> mutant A219; Streptococcal protein G<sub>B</sub> domain; Streptococcal protein G<sub>a</sub> domain; Protein G<sub>B</sub> mutant G311; E. coli hypothetical Yab; Bovine a-subunit of transducin; M. thermautrophicus CDC6; streptavidin; avidin; Taq polymerase; alkaline phosphatase; RNase; DNase; restriction enzymes; peroxidases; endo-1,4-beta glucanase; endo-1,3-beta-glucanase; chitinases; 10 beta and alfa glucosidases; beta and alpha glucuronidases; amylase; glucosyl-transferases; phospho-transferases; chloramphenicol-acetyl-transferase; beta-lactamase; luciferase; esterases; lipases; proteases; bacteriocines; antibiotics; enzyme inhibitors; growth factors; hormones; receptors; membranal proteins; nuclear proteins; transcriptional factors; translational factors or nucleic acid modifying enzymes.
- 15 17. The method according to claim 13, wherein the host cells includes cells from, Escherichia coli, Bacillus, Salmonella, Pseudomonas; Saccharomyces cerevisiae, Pichia pastoris, Kluveromyces, Candida, Schizosaccharomyces; or CHO cells.
- 20 18. A method for purifying a protein of interest from a fusion protein and separation therefrom, the method comprising:  
contacting a fusion protein comprising a prodomain protein linked to the protein of interest with an effective amount of subtilisin or a variant thereof under conditions suitable for the formation of a binding complex between the subtilisin or 25 variant thereof and the prodomain protein of the fusion protein;  
incubating the binding complex for a sufficient time for the subtilisin or variant thereof to cleave the protein of interest from the binding complex; and recovering the protein of interest.
- 30 19. The method according to claim 18, wherein the subtilisin has been modified to specifically bind to the protease prodomain fusion protein.
20. The method according to claim 19, wherein the subtilisin includes mutations Q2K, S3C, P5S, K43N, A73L, 75-83, E156S, G166S, G169A, S188P, Q206C, N212G,

K217L, N218S, T254A, Q271E, Y104A, G128S and at least one additional mutation at amino acid positions 32, 155 or 221.

21. The method according to claim 19, wherein the prodomain protein is a subtilisin prodomain and modified by replacing the P4 through P1 amino acids with amino acid sequences FKAM, FKAY or FKAQ.
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22. The method according to claim 21, wherein the protein of interest is staphylococcal Protein A<sub>B</sub> domain; Protein A<sub>B</sub> mutant A219; Streptococcal protein G<sub>B</sub> domain; Streptococcal protein G<sub>a</sub> domain; Protein G<sub>B</sub> mutant G311; E. coli hypothetical Yab; Bovine a-subunit of transducin; M. thermautrophicus CDC6; streptavidin; avidin; Taq polymerase; alkaline phosphatase; RNase; DNase; restriction enzymes; peroxidases; endo-1,4-beta glucanase; endo-1,3-beta-glucanase; chitinases; beta and alfa glucosidases; beta and alpha glucuronidases; amylase; glucosyl-transferases; phospho-transferases; chloramphenicol-acetyl-transferase; beta-lactamase; luciferase; esterases; lipases; proteases; bacteriocines; antibiotics; enzyme inhibitors; growth factors; hormones; receptors; membranal proteins; nuclear proteins; transcriptional factors; translational factors or nucleic acid modifying enzymes.
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23. The method according to claim 20, wherein the subtilisin is immobilized on a solid phase matrix.
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24. The method according to claim 21, wherein the prodomain of subtilisin is mutated to increase binding affinity of subtilisin to greater than  $10^9 \text{ M}^{-1}$ .
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25. The method according to claim 19, wherein the subtilisin includes mutations Q2K, S3C, P5S, K43N, A73L, 75-83, E156S, G169A, S188P, Q206C, N212G, K217L, N218S, T254A, Q271E, Y104A, G128S and at least one additional mutation at amino acid positions 32, or 221.
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26. The method according to claim 20, wherein the subtilisin is S189, S190, S194, S196, S197, or S198.
27. The method according to claim 25, wherein the subtilisin is S199, S201 or S202.

28. An assay method for detecting the presence of a substance of interest in a test sample comprising:

5 (a) incubating a test sample, which may contain a substance of interest, with a sufficient amount of a protease prodomain fusion protein, wherein the protease prodomain fusion protein comprises:

10 (i) a protease prodomain capable of binding with high affinity to a subtilisin or variant thereof, and

15 (ii) a second protein capable of binding the substance of interest,

under incubating conditions that allow for the binding of the substance of interest to the second protein;

15 (b) contacting the protease prodomain fusion protein used in step (a) to subtilisin or a variant thereof, wherein the subtilisin or a variant thereof is in solution in an amount effective to bind the fusion protein or immobilized on a solid phase to form a subtilisin/prodomain fusion protein binding complex;

20 (c) incubating the subtilisin/prodomain fusion protein binding complex for a sufficient time for the subtilisin or variant thereof to cleave the second protein from the binding complex;

25 (d) recovering the second protein bound to the substance of interest.

29. The method according to claim 28, further comprising introducing a detectable label capable of binding to the substance of interest; and determining the presence or absence of the label, to provide an indication of the presence or absence of the substance of interest in the test sample.

30. The method according to claim 29, wherein the detectable label is introduced before separation of the second protein from the binding complex or after the second protein is recovered.

31. The method according to claim 28, wherein the test sample is blood, urine, semen, saliva, mucus, tears, or vaginal secretions.

32. The method according to claim 31, wherein the substance of interest is an antibody.

33. The method according to claim 32, wherein the second protein is an antigenic receptor having affinity for the antibody.
- 5 34. The method according to claim 31, wherein the substance of interest is an antigen.
35. The method according to claim 34, wherein the second protein is an antibody having affinity for the antibody.
- 10 36. The method according to claim 28, wherein the subtilisin has been modified to specifically bind to the protease prodomain fusion protein.
37. The method according to claim 36, wherein the subtilisin includes mutations Q2K, S3C, P5S, K43N, A73L, 75-83, E156S, G166S, G169A, S188P, Q206C, N212G, K217L, N218S, T254A, Q271E, Y104A, G128S and at least one additional mutation at amino acid positions 32, 155 or 221.
- 20 38. The method according to claim 28, wherein the protease prodomain protein is a subtilisin prodomain and modified by replacing the P4 through P1 amino acids with amino acid sequences FKAM, FKAY or FKAf.
39. A drug delivery system comprising a subtilisin prodomain protein associated with a drug of interest to form a fusion product, wherein the fusion product is further complexed to a subtilisin or variant thereof to form a drug delivery complex.
- 25 40. The drug delivery system according to claim 39, wherein the drug of interest is conjugated to the subtilisin prodomain protein either directly or through a linker moiety.
- 30 41. The drug delivery system according to claim 39, wherein the drug of interest is slowly released from the drug delivery complex.
42. The drug delivery system according to claim 41, wherein the drug delivery product is included in a composition and administered parenterally, orally, topically or by

inhalation.

43. The drug delivery system according to claim 41, wherein the composition comprises a solid, gel, liquid or aerosol.

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44. The drug delivery system according to claim 41, wherein the subtilisin includes mutations Q2K, S3C, P5S, K43N, A73L, 75-83, E156S, G166S, G169A, S188P, Q206C, N212G, K217L, N218S, T254A, Q271E, Y104A, G128S and at least one additional mutation at amino acid positions 32, 155 or 221.

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45. The drug delivery system according to claim 41, wherein the subtilisin prodomain protein is modified by replacing the P4 through P1 amino acid residues with amino acid sequences FKAM, FKAY or FKAF.

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